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Simple Sequence Repeat Marker Associated with a Natural Leaf Defoliation Trait in Tetraploid Cotton

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Abstract

Cotton (*Gossypium hirsutum* L.) leaf defoliation has a significant ecological and economical impact on cotton production. Thus the utilization of a natural leaf defoliation trait, which exists in wild diploid cotton species, in the development of tetraploid cultivated cotton will not only be cost effective, but will also facilitate production of very high-grade fiber. The primary goal of our research was to tag loci associated with natural leaf defoliation using microsatellite markers in Upland cotton. The F_2 populations developed from reciprocal crosses between the two parental cotton lines—AN-Boyovut-2 ($2n = 52$), a late leaf defoliating type, and Listopad Beliy ($2n = 52$), a naturally early leaf defoliating type—demonstrated that the naturally early leaf defoliation trait has heritability values of 0.74 and 0.84 in the reciprocal F_2 population. The observed phenotypic segregation difference in reciprocal crosses suggested a minor cytoplasmic effect in the phenotypic expression of the naturally early leaf defoliation trait. Results from the Kruskal-Wallis (KW) nonparametric test revealed that JESPR-13 (KW = 6.17), JESPR-153 (KW = 9.97), and JESPR-178 (KW = 13.45) Simple sequence repeat (SSR) markers are significantly associated with natural leaf defoliation in the mapping population having stable estimates at empirically obtained critical thresholds ($P < .05$–$.0001$). JESPR-178 revealed the highest estimates ($P < .0001$) for association with the natural leaf defoliation trait, exceeding maximum empirical threshold values. JESPR-178 was assigned to the short arm of chromosome 18, suggesting indirectly that genes associated with natural leaf defoliation might be located on this chromosome. This microsatellite marker may have the potential for use to introgress the naturally early leaf defoliation quantitative trait loci (QTL) from the donor line Listopad Beliy to commercial varieties of cotton through marker-assisted selection programs.

In cotton (*Gossypium hirsutum* L.), leaf defoliation is one of the important components of worldwide cotton management for producing cotton fiber with superior quality. Defoliation enhances and speeds boll opening, accelerates the harvesting time, improves fiber grades, and often reduces boll rot. Proper timing of defoliation is challenging, as reduced yield and fiber quality may be caused by defoliation at the incorrect time. Improper use of defoliating agents and time of crop termination significantly affect fiber length, length uniformity, micronire, and fiber strength (Larson et al. 2002). The harmful effects of premature defoliation on yield and fiber quality have also been studied (Snipes and Baskin 1994). Thus determination of the proper defoliation time for each environment is very important. In addition, defoliating chemicals are very costly and can be hazardous to the environment through accumulation of chemicals in the soil and pollution of drinking water. One of the approaches to overcome these challenges in cotton defoliation is the development of naturally early leaf defoliating cotton varieties through introgression of the trait from certain diploid *Gossypium* species. Several wild diploid cotton species have the naturally early leaf defoliation trait [e.g., *Gossypium thurberi*, *Gossypium aridum*, *Gossypium lobatum*, and *Gossypium laxum* defoliate leaves close to the time of boll opening (Fryxell 1975)].

Cotton is the primary economic resource in Uzbekistan. The development of natural leaf defoliating varieties is one

of the important goals of the cotton breeding program in the republic. Scientists have studied and reported an early leaf defoliation trait in the cotton breeding germplasm of Uzbekistan. Barhatova (1990) studied the early leaf defoliation trait that exists in *G. hirsutum* breeding cultivars/lines and reported the inheritance and correlation of this trait with other agronomic traits in cotton, making several reciprocal crosses between late, middle, and early leaf defoliating cultivars/lines of Uzbekistan. However, the genetics of a naturally early leaf defoliation trait in wild cotton was not studied. Hence scientists have developed a naturally early leaf defoliating Upland cotton line named Listopad Beliy (white leaf defoliant, AADD, $2n = 2x = 52$), from trispecies crosses between *G. thurberi* (D_1D_1, $2n = 2x = 26$, natural defoliant) × *G. harknessii* (D_2D_2, $2n = 2x = 26$, wilt resistant) × *G. hirsutum* (variety Tashkent-1, AADD, $2n = 2x = 52$), using an integrated approach of chromosome doubling via colchicine and conventional breeding methods at the Institute of Genetics and Plant Experimental Biology (IGPEB), Uzbekistan (Rizaeva et al. 2001). This line, with earliness, drought and wilt resistance, and a natural leaf defoliation trait, has been used as parental donor material in breeding programs to introgress the natural leaf defoliation trait into many commercial varieties in Uzbekistan. Understanding the genetic inheritance of the naturally early leaf defoliation trait of wild diploid cottons and identification of linked DNA markers should expedite the introgression of this important trait. The objectives of this work were to identify markers associated with leaf defoliant genes and to identify the chromosomal location of single sequence repeat (SSR) markers linked to the trait.

Materials and Methods

Plant Materials

Listopad Beliy ($2n = 2x = 52$, AADD), a natural leaf defoliating line derived from a trispecies hybrid (*G. thurberi* [$2n = 2x = 26$, D_1D_1] × *G. harknessii* [2n = $2x = 26$, D_2D_2] × *G. hirsutum* var. Tashkent-1 [$2n = 2x = 52$, AADD]), was crossed with late leaf defoliating cultivar *G. hirsutum* var. AN-Boyovut-2 ($2n = 2x = 52$, AADD). AN-Boyovut-2 is a widely grown, salt resistant commercial line in Uzbekistan, obtained from extensive selection of biotypes of Tashkent variety (developed from the cross between *G. hirsutum* var. C-4727 and *G. hirsutum* ssp. *mexicanum* with consequent backcrossing of F_1 with C-4727) in saline regions of Uzbekistan. These two homozygous cotton parental lines differ significantly in their natural leaf defoliating percentage, as Listopad Beliy defoliates 70–100% of its leaves during boll opening time, whereas AN-Boyovut-2 defoliates 17–40% of its leaves under the Tashkent growing environment (Figure 1 and Table 1).

F_1 plants from AN-Boyovut-2 and Listopad Beliy reciprocal crosses were grown in the field to generate F_2 seeds. A total of 198 and 189 F_2 plants from reciprocal crosses were grown under field conditions in 1999. In this growing season the average temperature in Tashkent from August 1 to September 5 was 27°C with an average humidity of 44.33% (these values were calculated from the archive of the meteorology center; available at http://meteo.infospace.ru/wcarch/html, visited April 2, 2005). The individual F_2 plants from the reciprocal crosses were analyzed for the total natural leaf defoliation percentage and other agronomic traits under field conditions. Measurements of leaf defoliation were based on the percent (20–100%) of total plant leaves lost during the 2 weeks following first boll opening (August 20–September 5). The leaf defoliation percentages of individual plants were measured by calculating the number of green leaves that remained on the plant and the number of leaf petiole sites or so-called abscission zones remaining after leaf defoliation under field conditions. The sum of green leaves and abscission zones gave the total plant leaf number from which the defoliation percentage was calculated. Measurements of leaf defoliation were conducted at least three times during the 2-week period. All plants were measured for leaf defoliation on the same day. During the 2-week measurement period, plants with naturally early leaf defoliation properties, such as Listopad Beliy, lost up to 100% of their leaves, demonstrating significant variations that were enough to differentiate genotypes and record trait values.

The F_2 plants from each cross were scored for leaf defoliation percentage on the main stem, sympodia, and monopodia, and the percentage of leaf defoliation on the entire plant was used for genetic inheritance analysis. Since leaf defoliation percentages in F_2 plants were continuous, F_2 plants were categorized into three groups based on the percentage of defoliated leaves: 17–40%, 41–70%, and 71–100% (Figure 2). Plants that had 17–40% leaf defoliation were considered as representative of the late leaf defoliating parent, since AN-Boyovut-2 defoliates in the range of 17–40% of its leaves close to boll opening, depending on the environment (Figure 1 and Table 1). The group with 40–70% leaf defoliation was in the middle range, since F_1 plants defoliate an average 54–60% of total leaves; and the group with 71–100% leaf defoliation was considered to be early leaf defoliating, similar to the leaf defoliation range of Listopad Beliy (Figure 1).

The F_2 plants from the cross between Listopad Beliy as maternal parent and AN-Boyovut-2 as paternal parent were self-pollinated up to the F_5 generation to obtain recombinant inbred lines (RILs) homozygous for the natural leaf defoliation trait, since the natural leaf defoliation trait is expressed more in this population (Figure 2). A total of 102 F_5 plants were grown and phenotypically evaluated under field conditions in the 2002 growing season, where the average temperature from August 1 to September 5 was 26.7°C and the average humidity was 37% (http://meteo.infospace.ru/wcarch/html, visited April 2, 2005). Further, 66 F_5 plants from the cross between Listopad Beliy as maternal parent and AN-Boyovut-2 as paternal parent were selected from a group of 102 F_5 plants for identification of DNA markers associated with this trait. The 66 plants selected represented several in each parental phenotype and the middle phenotype for the natural leaf defoliation trait (Figures 2 and 3). The total plant leaf defoliation percentage of the 66 individual

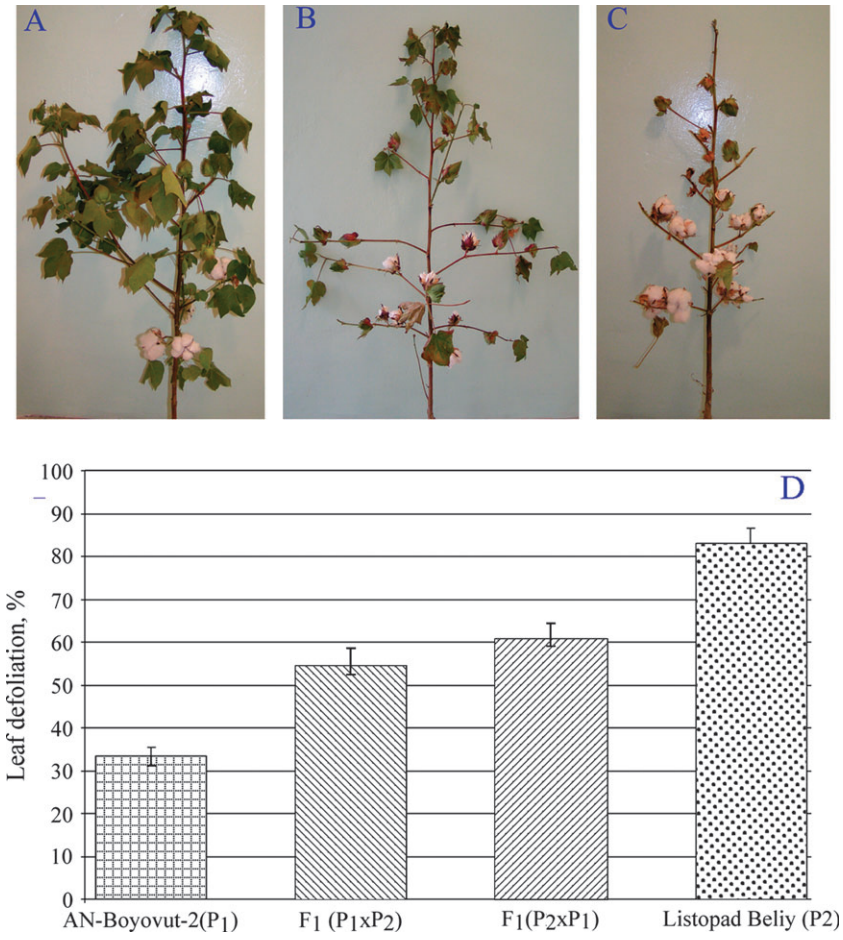

Figure 1. The phenotypes associated with leaf defoliation of (A) AN-Boyovut-2, (B) F_1, (C) Listopad Beliy, and (D) the bar graph representing the average trait values of plants at the time of boll opening (statistical values for leaf defoliation for these plants are given in Table 1).

F_5 plants obtained in the 2002 growing season was used for marker and trait correlation. Young cotton leaves from each of the 66 plants were collected and used for genomic DNA preparations.

Genotyping and Linkage Analysis

Genomic DNA samples were extracted from collected cotton tissues following the method of Dellaporta et al. (1983), with minor modifications for frozen cotton leaf tissues. SSR or microsatellite markers from JESPR (Reddy et al. 2001), CM (Connell et al. 1998; Reddy and Pepper unpublished), BAC-derived SSRs (Yu et al. 2002, 2004; available through CottonDB at http://cottondb.tamu.edu, visited April 2, 2005), MGHES (Qureshi et al. 2004), and MGAES (Saha et al. unpublished) were utilized to tag the trait of interest. Unpublished SSR marker collections are available at the specific laboratories where the markers have been developed.

Table 1. The statistical values for the leaf defoliation trait in parental, F_1, and F_2 reciprocal crosses

Plant type	Number of plants	Minimum value (%)	Maximum value (%)	Median	Mean	Std. Dev.	SE
AN-Boyovut-2 (P_1)	10	17.3	39.58	34.57	33.32	6.7	2.12
Listopad Beliy (P_2)	11	69.76	100	78.04	83.16	11.61	3.501
F_1 from $P_1 \times P_2$	7	37.73	70.58	51.28	54.54	10.66	4.031
F_1 from $P_2 \times P_1$	8	41.21	75	63.14	60.71	10.53	3.724
F_2 from $P_1 \times P_2$	198	18.18	100	77.15	74.24	24.87	1.767
F_2 from $P_2 \times P_1$	189	40	100	73.68	77.38	19.39	1.411
F_5 from $P_2 \times P_1$	102	22.2	100	62.70	64.89	21.02	2.082

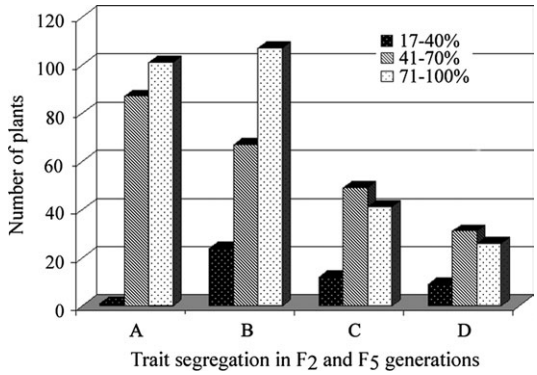

Figure 2. Phenotypic segregation of the naturally early leaf defoliation in F_{2-5} generations: (A) 189 F_2 plants from the cross between Listopad Beliy and AN-Boyovut-2 (1:87:101); (B) 198 F_2 plants from the cross between AN-Boyovut-2 and Listopad Beliy (24:67:107); (C) 102 F_5 plants of the cross between Listopad Beliy and AN-Boyovut-2 (12:49:41); and (D) 66 F_5 individuals selected for molecular genotyping (9:31:26). Plants have been grouped as 17–40%, 41–70%, and 71–100% total plant leaf defoliation phenotypes.

Microsatellite genotyping analyses were performed following the methods of Reddy et al. (2001).

Primer pairs for SSRs were amplified using a hot-start polymerase chain reaction (PCR) protocol with genomic DNA of the parental lines as template. The primers amplifying polymorphic fragments between parents were used to screen the genomic DNA of the selected F_5 individuals. Amplification reactions were performed in 25 µl volumes containing 2.2 µl 10× PCR buffer with $MgCl_2$, 0.4 µl BSA, 0.2 µl 25 mM dNTP mix, 2.0 µl of 25 ng/µl of each reverse and forward primer, and 1 µl of 25 ng/µl template DNA. Then 0.2 U *Taq* DNA polymerase (Sigma, St. Louis, MO; or Orbigen, San Diego, CA) was added to the reaction at the annealing temperature of the first cycle. Amplifications were carried out with a first denaturation at 95°C for 3 min followed by 45 cycles of 94°C for 1 min, 50°C for 1 min (annealing), and 72°C for 2 min (extension). A final 5-min extension at 72°C was then performed. Polymorphism of microsatellite amplification products was revealed using polyacrylamide and agarose gel systems.

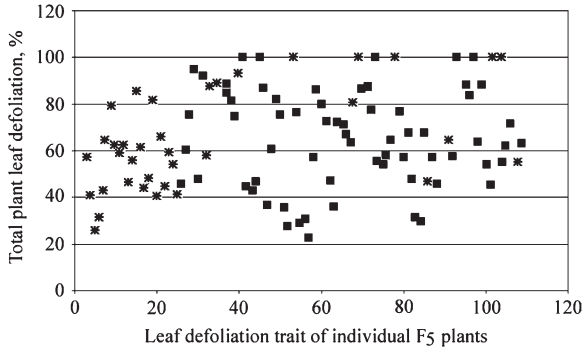

Figure 3. The leaf defoliation trait distribution of individual F_5 plants: ✱ not genotyped individuals; ■ genotyped individuals.

In the polyacrylamide system, samples were electrophoresed at 20 V/cm in a megagel dual 42 cm high × 50 cm wide × 1 mm thick adjustable vertical system gel rig (CBS Scientific, Del Mar, CA) containing 6% polyacrylamide (29:1) in 1× TBE buffer, then visualized after staining with ethidium bromide. In the agarose system, samples were electrophoresed on a 16 cm long horizontal gel (Stratagene, La Jolla, CA) containing a mixture of 2% agarose and 2% Metaphor agarose (FMC, Philadelphia, PA) at 5.3 V/cm in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8) with the buffer chilled to 4°C. Gels were stained with ethidium bromide and photodocumented using an Alpha Imager 3400 (Innotech, San Leandro, CA).

Genotypic data were correlated with the phenotypic data using the Kruskal-Wallis nonparametric test functions of MapQTL 4.0 software (Van Ooijen et al. 2002) and simple marker regressions were performed using QGene 3.0 software (Nelson 1997). The individuals of the F_5 population that were not genotyped were also been included in single marker analysis (SMA), indicating "missing" data for genotypes. The permutation analyses with 1000 and 10,000 shuffles were carried out to determine stable estimates for markers associated with leaf defoliation quantitative trait loci (QTL) (Churchill and Doerge 1994). The entire permutation analysis using QGene was repeated 34 times and the average critical threshold values from these tests were calculated and used for estimation of marker associations.

Chromosomal Localization of Trait-Associated SSRs

The overall method of identifying the chromosomal location of the leaf defoliant locus (loci) for linked SSR markers was used as per the methodology of Karaca et al. (2002). DNA from samples of young leaves of individual monosomic chromosome or monotelodisomic chromosome (*G. tomentosum*) substitution stocks of TM1 (*G. hirsutum*) from BC_0F_1 plants were used to assign the specific molecular markers to the appropriate chromosome. If the SSR marker linked to the natural defoliation leaf locus was located on the specific *G. tomentosum* chromosome or chromosome arm being introgressed into the monosomic or monotelodisomic chromosome substitution stock, then the sample would exhibit only the respective *G. tomentosum* bands and would lack the allelic TM1 band. The mapping logic is simple and direct: if a DNA marker is absent in a plant missing a known part of a single chromosome, but is present in a plant with a complete chromosome, then it can be logically inferred that the DNA marker is located in that segment of the missing chromosome. Leaf samples from individual plants of the aneuploid chromosome substitution lines (BC_0F_1) for the chromosomes of 1, 2, 3Sh, 3Lo, 4, 5Lo, 6, 7, 8, 9, 10, 11Lo, 12Sh, 14Lo, 15Sh, 16, 18, 18sh, 20, 22Sh, 22Lo, 25, and 26Sh were used for genomic DNA extraction. Capillary electrophoresis on an ABI Prism 310

Genetic Analyzer (Applied Biosystems, Foster City, CA) was used for chromosomal localization experiments following the methods of Karaca et al. (2002).

Results

Trait Segregation

F_1 plants from a cross between Listopad Beliy as maternal parent and AN-Boyovut-2 as paternal parent defoliated an average 60.7% of the total plant leaves. The reciprocal F_1 plants defoliated an average 54.53% of the total leaves, demonstrating a moderate level of leaf defoliation (incomplete dominance) in both crosses (Table 1 and Figure 1). The minor difference observed between the F_1 progeny of two reciprocal crosses could be due to environmental variation or a minor cytoplasmic effect.

Further, F_1 plants from these reciprocal crosses were self-pollinated to obtain F_2 seed and individual F_2 plants were scored for total leaf defoliation percentage as well as leaf defoliation rate on the main stem, sympodia, and monopodia close to the time of boll opening. We observed that in F_2 plants, leaf defoliation always began with the main stem, and the rate of defoliation was higher on the main stem than on the sympodia or monopodia (data not shown). The segregation pattern of 198 F_2 plants from the cross of AN-Boyovut-2 as maternal parent and Listopad Beliy as paternal parent showed a wide range of leaf defoliation phenotypes (18–100%). The results showed that the phenotypic distribution of F_2 plants from this cross was skewed toward the natural leaf defoliation trait, and the phenotypic ratio of the F_2 plant groups (17–40%, 41–70%, and 71–100%) was 24:67:107 (Figure 2). This observed segregation pattern is distorted from the Mendelian segregation ratio for monogenic incomplete dominance (1:2:1) as the chi-square value ($\chi^2 = 91.27$) was significant at $\alpha = 0.001$ with two degrees of freedom.

The leaf defoliation range of 189 F_2 plants from the cross between Listopad Beliy as maternal parent and AN Boyovut-2 as paternal parent also showed a range of leaf defoliation from 40% to 100%. This range was a little higher than the reciprocal cross (18–100%), suggesting some influence of cytoplasmic and environmental factors. The grouped data from F_2 plants of this cross showed a skewed distribution pattern toward the leaf defoliating parent, and the phenotypic ratio of the F_2 plant groups (17–40%, 40–70%, and 71–100%) was 1:87:101 (Table 1). In this cross, the observed segregation also significantly deviated from the expected segregation ratio for incomplete dominance (1:2:1), with $\chi^2 = 107$ being highly significant at $\alpha = 0.001$ with two degrees of freedom ($\chi^2 = 13.82$). The observed differences in the leaf defoliation ratio between the two groups of F_2 plants of the reciprocal crosses demonstrated that inheritance of the natural leaf defoliation trait is complex and that segregation possibly has a non-Mendelian component and is probably a quantitative trait controlled by polygenes.

We also calculated the broad sense heritability (H^2) based on variations obtained from parents, and F_1 and F_2 progenies of reciprocal crosses between AN-Boyovut-2 and Listopad Beliy (Table 1). In the cross of AN-Boyovut-2 as maternal parent and Listopad Beliy as paternal parent, the broad sense heritability was 0.84, demonstrating 84% of trait variation was genetic and 16% of trait variation was environmental. The heritability value obtained from the cross between Listopad Beliy as maternal parent and AN Boyovut-2 as paternal parent was equal to 0.74, showing 74% of trait variation was due to genetic factors and the remaining 26% was environmental. The obtained difference in heritability values of reciprocal crosses might also be due to minor cytoplasmic effects coming from the Listopad Beliy parent.

Further, for the purpose of developing a natural leaf defoliating cotton cultivar, F_2 plants from the cross between Listopad Beliy as maternal parent and AN Boyovut-2 as paternal parent have been self-pollinated for five generations to obtain homozygous plants for this trait, since this combination showed a greater expressed phenotype for the natural leaf defoliation trait. We measured the leaf defoliation percentages of individual F_5 plants during the 2-week period. F_5 plants defoliating 100% of their total leaves (like Listopad Beliy) lost 61% of total leaves by August 20 and 100% by September 5, demonstrating the significant variation in the total natural leaf defoliation percentage over the 2-week period. We observed that F_5 plants defoliated leaves in the range of 22–100%. This range was different from the leaf defoliation range of F_2 plants (40–100%) of the cross between Listopad Beliy as maternal parent and AN Boyovut-2 as paternal parent, suggesting the influence of environmental factors. Moreover, this difference could be due to the appearance of more parental-type genotypes after many generations of self-pollination (Abdullaev A, personal communication). These plants of the F_5 generation were also used for identification of molecular markers associated with the natural leaf defoliation trait. We selected 66 F_5 plants from the 102 plants in the F_5 population representing extreme and a number of midrange phenotypes of leaf defoliation (Figures 2 and 3) and genotyped with available SSR markers in our laboratory for the purpose of identifying DNA markers associated with this important trait.

Marker Association

A total of 176 JESPR microsatellite primer pairs (Reddy et al. 2001) were screened against parental lines Listopad Beliy and AN-Boyovut-2 in an effort to find polymorphic markers. One hundred forty-one JESPR primer pairs (80%) produced reliable PCR products, however, only seven (5%) (JESPR-6, JESPR-13, JESPR-178, JESPR-56, JESPR-101, JESPR-153, JESPR-251) detected polymorphism between Listopad Beliy and AN-Boyvout-2.

Primer pairs for 96 BAC-derived SSRs of TM1 (Yu et al. 2002, 2004) have also been screened over parental lines, of which 86 (89%) produced PCR products. Of these 86 BAC-derived SSRs, four markers (5%) detected potential polymorphisms between these two parents (TMB0189, TMB0436, TMB0004, and TMB0283). In addition, we tested 75 EST-SSRs of *G. hirsutum* MGHES markers (Qureshi et al. 2004), of which 67 (89%) amplified parental genomic DNA;

Table 2. SSR markers screened over parental lines

Marker name	Amplified	Not amplified	Polymorphic	Total
TMB	86	10	4	96
JESPR	141	35	7	176
CM	6	0	0	6
MGHES	67	8	0	75
MGAES	101	98	0	199
Total	401 (72.6%)	151 (27.4%)	11 (3%)	552

however, no polymorphism was detected between parental lines. One hundred ninety-nine EST-SSRs of *G. arboreum* MGAES markers (Saha et al. unpublished), have also been screened, of which 75% amplified parental DNA, yet no polymorphism was detected. To increase polymorphic marker numbers, six CM (Connell et al. 1998; Reddy and Pepper, unpublished) microsatellites have also been tested, all of which were monomorphic between parents. In total, 11 SSR markers (3%) detected polymorphisms in parents, indicating narrowness of the genome of parental lines (Table 2).

To determine the association of polymorphic SSRs with the natural leaf defoliation trait, 66 F_5 individual plants selected for extreme opposite and middle range phenotype for the leaf defoliation trait were genotyped with these seven JESPR and four BAC-SSRs primer pairs. The genotypic data were correlated with the leaf defoliation percentages in SMA using the Kruskal-Wallis nonparametric test of MapQTL and simple regression analysis using QGene. The Kruskal-Wallis rank sum test orders individuals according to phenotype and then sorts them, one locus at a time, by marker genotype (Lehmann 1975). We first used the Kruskal-Wallis test to increase the likelihood of determining the significant QTLs because of the few polymorphic markers found between parents. We also included the remaining not-genotyped F_5 individuals in the analyses indicating "missing" data for genotypes. The SMA using the Kruskal-Wallis test of MapQTL revealed that 4 SSR markers out of 11 [JESPR-56 ($P = .05$), JESPR-153 ($P = .01$), JESPR-13 ($P < .001$), and JESPR-178 ($P < .001$)] were significantly associated with leaf defoliation QTL (Table 3). The remaining markers tested were not significant (data not shown). Moreover, the simple marker regression analysis performed using QGene determined that three SSR markers (JESPR-13, JESPR-153, and JESPR-178) were associated with the leaf defoliation QTL with *P*-values from .03 to less than .001 (Table 3). These results showed that at least these three SSR markers might be informative in detecting the natural leaf defoliation trait in cotton. These SSR markers segregated as codominant markers in the mapping population, and chi-square segregation analysis demonstrated that SSR markers associated with the natural leaf defoliation trait in SMA significantly deviated from expected marker segregation ratios for codominant (1:2:1) markers. As expected, the occurrence of heterozygotes was low (e.g., 9% at the JESPR-178 locus), since the mapping population had been self-pollinated for four generations and the population selectively genotyped.

Table 3. Association of SSR markers with fiber development QTL in single marker analyses

Marker name	KW (df = 1)	*P*	*F*	R_2
JESPR-178	13.45***	>.0001	18.5	0.23
JESPR-13	9.97***	.03	4.99	0.072
JESPR-56	3.16*	.08	3.17	0.049
JESPR-153	6.17**	.007	7.80	0.11

*, **, *** Kruskal-Wallis (KW) nonparametric test significant at the 0.05, 0.01, and <0.01 levels, respectively, obtained in SMA using MapQTL.

P value, *F* statistics, correlation coefficient squared (R^2) obtained by simple marker regression using QGene software.

Although the results of SMA demonstrated that these SSR markers are the potential loci in cotton contributing to the natural leaf defoliation trait, selective genotyping (Darvasi and Soller 1992) and small sample size (Beavis 1994) are directly concerned with the estimation of QTLs. Because of the small sample sizes and distributional properties of quantitative traits, the critical threshold values should be estimated and compared with marker statistics values to determine stable estimates for marker association (Churchill and Doerge 1994). The permutation analysis was proposed to detect stable estimates for the association of markers with a particular marker (comparison-wise) and all markers in a genome (experiment-wise) and the significant critical values at various probabilities calculated based on the number of shuffles used. At a minimum, permutation analysis with 1000 shuffles should be performed for estimating critical values at $\alpha = 0.05$, or shuffle numbers should be increased up to a maximum of 10,000 for estimating critical threshold values at more precise and significant ($\alpha = 0.01$) probabilities (Churchill and Doerge 1994).

The permutation analyses with 1000 shuffles of four significant leaf defoliation associated SSR markers demonstrated that three SSR markers (JESPR-13, JESPR-153, and JESPR-178) were significant at the $\alpha = 0.05$ empirical threshold point, where we observe a significant difference of unshuffled marker *F*-statistics values exceeding the average critical threshold values of permutation. Further, JESPR-153 and JESPR-178 markers were determined to have a stable association at $\alpha = 0.01$ critical threshold values of 1000 shuffles, and JESPR-178 was stable at the 100% threshold (F_{max}), exceeding the average empirical value of the permutation test (Table 4). We compared the marker *F*-statistics obtained in simple marker regression with genome-wise threshold values (experiment-wise) and observed that only marker JESPR-178 significantly exceeded the average critical experiment-wise values at maximum probability (F_{max}). This demonstrated the significance of JESPR-178 in the detection of the natural leaf defoliation QTL in the cotton genome (Table 4). The results of the permutation analysis with 10,000 shuffles also demonstrated that JESPR-13, JESPR-153, and JESPR-178 had stable estimates at $\alpha = 0.05$. JESPR-153 and JESPR-178 were stable at $\alpha = 0.01$ and JESPR-178 was stable at the 100% critical threshold of permutation analysis (Table 4).

Table 4. The results of permutation analysis using QGene software

Marker	*F* from single marker regression	Permutation analysis with 1000 shuffles			Permutation analysis with 10, 000 shuffles		
		95th percentile[a]	99th percentile[a]	F_{max}[a]	95th percentile[a]	99th percentile[a]	F_{max}[a]
JESPR-178	18.5	3.99 ± 0.04	6.87 ± 0.09	14.67 ± 0.57	4.01 ± 0.01	7.04 ± 0.03	18.24 ± 0.5
JESPR-13	4.99	3.94 ± 0.05	6.99 ± 0.11	12.17 ± 0.48	3.99 ± 0.01	7.07 ± 0.03	18.44 ± 0.4
JESPR-56	3.17	3.96 ± 0.05	6.88 ± 0.07	13.70 ± 0.59	3.98 ± 0.01	7.05 ± 0.03	18.28 ± 0.5
JESPR-153	7.80	3.99 ± 0.04	7.02 ± 0.09	13.37 ± 0.44	4.00 ± 0.01	7.10 ± 0.04	18.65 ± 0.5
Experiment-wise		14.09 ± 0.34	14.09 ± 0.34	17.01 ± 0.54	18.86 ± 0.3	18.86 ± 0.3	21.86 ± 0.4

[a] The average empirical values from 34 permutation analyses.

Chromosomal Localization

Amplification of DNA from the cytogenetic stocks with the JESPR-178 primer pair showed that the TM-1 parental marker was absent in the aneuploid hybrid F_1 plant missing chromosome 18 from TM-1 and the F_1 hybrid plant missing the short arm of chromosome 18 from TM-1. These results demonstrated directly that the JESPR-178 marker, and indirectly that a significant genetic component controlling the natural leaf defoliation trait loci, is located in the short arm of chromosome 18 (Figure 4).

Discussion

The introgression of a naturally early leaf defoliation trait from a wild diploid *Gossypium* species into Upland cotton is one of the important goals of cotton breeding programs at Uzbekistan. This may also be useful in other cotton-producing countries as well. Identification of the genetic mode of inheritance and tagging of the natural leaf defoliation trait in cotton with molecular markers will help breeders to improve the cotton germplasm.

The physiological aspects of organ abscission/defoliation have been studied in detail in several plant families and several factors are now known to play a significant role in the general abscission process (Taylor and Whitelaw 2001). In general, abscission occurs in the "abscission zone" due to a change in the phytohormone balance (ethylene/auxin/abscisic acid) and hydrolytic enzyme (cellulase and polygalactouronase) activity in plant organs, including the leaf petioles (Hong et al. 2000; Kalaitzis et al. 1997; Taylor et al. 1990). Several members of gene families that have a major impact on plant organ abscission (such as cellulase, polygalactouronase, ethylene receptor gene family, MADS-box, G-box, and VIHID protein) also have been cloned and characterized (Fernandez et al. 2000; Liljegran et al. 2000; Mao et al. 2000; Roberts et al. 2000; Schumacher et al. 1999). These studies showed that abscission in plants is an environmentally dependent and organ-specific process resulting from a complex interconnected network of gene interactions.

There are a number of reports in the former Soviet Union on the development of naturally early leaf defoliating varieties of *G. hirsutum* and *G. barbadense* based on chemical mutagenesis (Abdullaev and Khojimuradova 1981; Egamberdiev and Daminov 1982; Fursov 1976; Maksimenko 1951, 1955; Rakhimboev 1986; Rizaeva 1983, 1996; Sadikov 1981, 1983). Most of these reports presented the early leaf defoliation trait that existed in breeding materials of tetraploid cottons and were descriptive regarding trait inheritance. Barhatova (1990) studied detail genetic inheritance of early leaf defoliation in breeding cultivars of *G. hirsutum*, making crosses between a number of Uzbekistan cultivars for late (e.g., 175-F and L-1233, defoliating 34–40% of leaves), middle (L-426 and L-955, defoliating 64% of leaves), and early (142-F and L-3729, defoliating 73–85% of leaves) leaf defoliation properties. Barhatova (1990) reported incomplete dominance of the early leaf defoliation trait in the F_1 generation of the crosses between breeding cultivars/lines and demonstrated the transgressive segregation pattern of this trait in the F_2 generation. The genetic inheritance estimated in that study was equal to the average of 0.48, and polygenic regulation of this trait has been reported. However, there are no reports on the utilization and inheritance of a natural leaf defoliation trait from wild cotton or on molecular tagging of loci associated with a natural leaf defoliation trait in cotton.

The development of a unique natural leaf defoliating trigenomic (*G. thurberi* × *G. harknessii* × *G. hirsutum* var. Tashkent-1) hybrid line (Listopad Beliy) was a source to study this important trait. The F_1 plants of reciprocal crosses between a naturally defoliating line, Listopad Beliy ($2n = 52$, AADD), and a commercial Uzbek variety, AN-Boyovut-2 ($2n = 52$, AADD), revealed incomplete dominance of the natural leaf defoliation trait. In the F_2 generation of these reciprocal crosses, we observed that the naturally early leaf defoliation trait had complex QTL inheritance that probably involves several genes and may include cytoplasmic factors. The difference in the range of leaf defoliation percentages in F_1 plants of reciprocal crosses was small. However, the segregation ratios and calculated broad sense heritability values (0.74 and 0.84) were different in the reciprocal F_2 populations, demonstrating at least 16% of the phenotypic variation is due to environmental factors. Moreover, our result indicated that leaf defoliation might be influenced by nuclear and cytoplasmic interactions. Barhatova (1990) also observed a cytoplasmic effect on the early leaf defoliation trait in the reciprocal crosses of various breeding cultivars. Other morphological mutants in cotton are also controlled by both cytoplasmic and nuclear interaction. For example, Karaca et al. (2004) reported that the Cyt-V mutant in cotton is controlled by cytoplasmic and nuclear interactions.

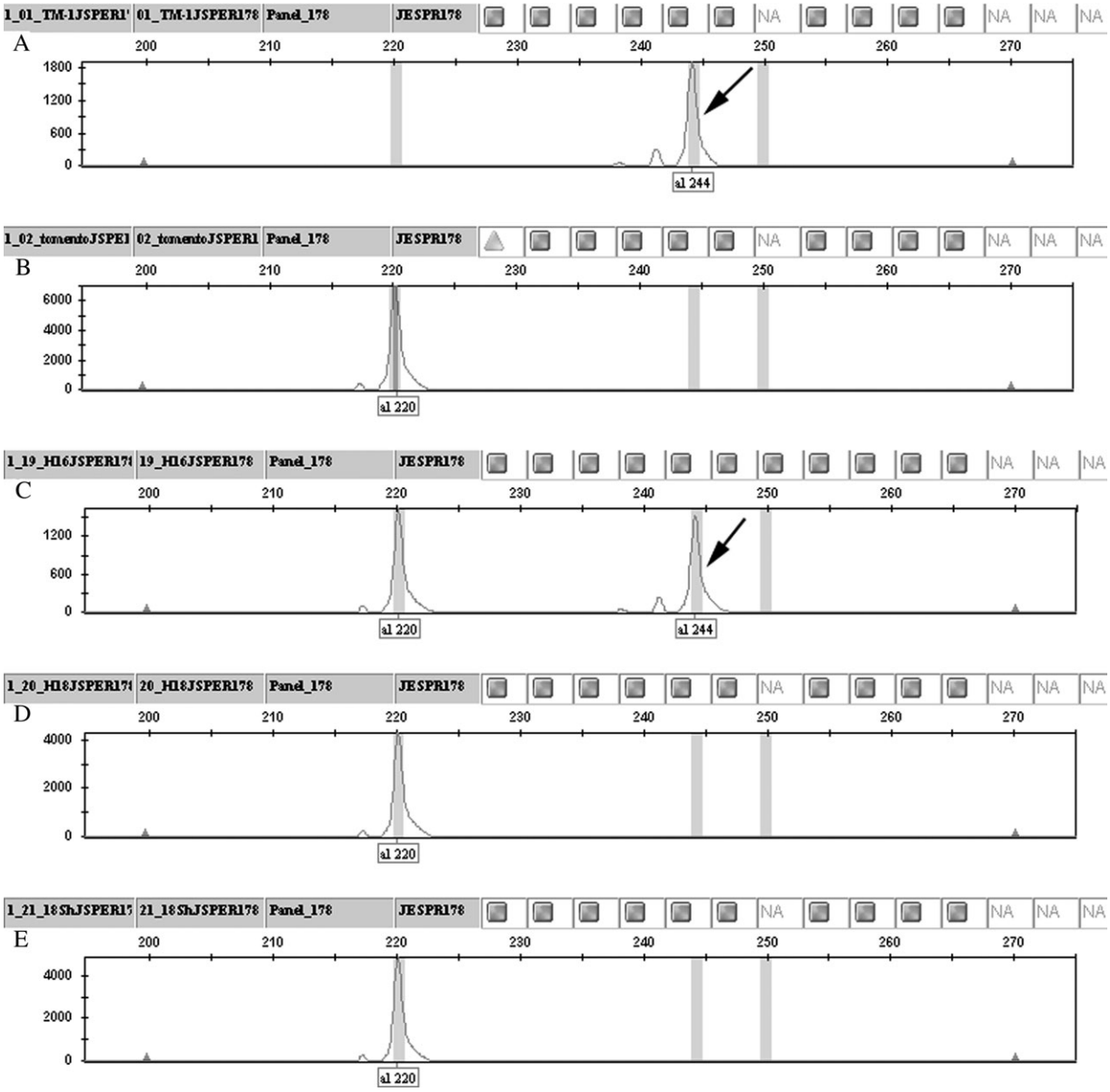

Figure 4. Capillary electrophoresis results showing the chromosomal positions of JESPR-178. (A) TM1; (B) *G. tomentosum*; (C) F_1 of TM1 and *G. tomentosum*; (D) cytogenetic line with substitution of chromosome 18; (E) cytogenetic line with substitution of short arm of chromosome 18.

Naturally early leaf defoliation also might contribute to cotton production since cotton bolls of naturally defoliating lines mature early. Thus, although we did not study the boll opening range in the reciprocal crosses, one can surmise that phenotypic expression of the natural leaf defoliation trait in cotton could also be associated and interact with earliness genes in cotton. The relationship between leaf defoliation and maturity needs further study, although there was no significant correlation recorded between early leaf defoliation and early maturation, fiber quality, and a number of other morphological characteristics of cotton in previous studies (Barhatova 1990).

One of the challenges in the application of marker-assisted selection in cotton breeding programs is the limited number of molecular markers associated with important traits. Microsatellite or SSR markers are powerful, portable, and easy-to-use molecular markers to tag traits of interest in cotton because of their informativeness and random distribution in a genome (Karaca et al. 2002; Qureshi et al. 2004; Reddy et al. 2001). The SSR polymorphism between the two parents, AN-Boyovut-2 and Listopad Beliy, was very low (3%), indicating the narrow genetic difference between the two parents. In the development of Listopad Beliy (the trigenomic hybrid with high natural leaf defoliation trait),

the Tashkent-1 cultivar was used. AN-Boyovut-2, the other parent in this study, was also derived from extensive selection of Tashkent variety biotypes in saline conditions. This also explains the genome-wise similarity of the parental lines, AN-Boyovut-2 and Listopad Beliy. Therefore any polymorphic marker is important within this narrow genetic base.

Naturally early leaf defoliation QTL association efforts using SSR markers determined that at least tree SSR markers—JESPR-178, JESPR-153, and JESPR-13—were significantly associated($P = .05$–$.01$) with the natural leaf defoliation trait in selected F_5 recombinant inbred lines segregating for leaf defoliation trait. Association of the JESPR-178 marker was stable at the empirically obtained critical threshold value ($P = .00001$), demonstrating its power in detecting the natural leaf defoliation QTL in cotton. In reality, the detection of QTL having reasonable power in breeding populations requires large sample sizes (Soller et al. 1976). For identification of single QTL regions in a genome with markers, the selective-genotyping procedure has been proposed for experimental populations (Darvasi and Soller 1992; Lander and Botstein 1989), where two extremes of the population are used for mapping purposes. Although this approach has several limitations regarding the power of linked markers, it is appropriate in tagging single QTLs (Darvasi 1997). In our study we selected the two extreme phenotype individuals with very late leaf defoliation percentage (22–40%) and high leaf defoliating (70–100%) genotypes. Moreover, we also genotyped midrange leaf defoliating individuals (40–70%), which gives power to detected marker associations. The JESPR-178 SSR marker was associated with the leaf defoliation QTL with a high probability ($P < .0001$) when compared with genome-wise empirical threshold values, demonstrating its potential usefulness in marker assisted selection (MAS) programs to transfer a naturally early leaf defoliation trait from donor line Listopad Beliy to commercial cultivars of cotton.

Results from the analysis of two aneuploid chromosome substitution lines missing chromosome 18 and the short arm of chromosome 18 showed that JESPR-178, linked to naturally early leaf defoliating genes, is absent in both cases. This result confirmed indirectly that a gene related to naturally early leaf defoliation in cotton is located on the short arm of chromosome 18. Several morphological trait genes associated with flower color and open bud were located on linkage group XII or chromosome 18 (Endrizzi et al. 1985). Zhang et al. (2002) reported that chromosomes 5 and 18 form homologous linkage groups based on molecular marker analysis. Rong et al. (2004) observed seven duplicated loci on the same chromosome 18, suggesting this chromosome has undergone extensive duplication. Lacape et al. (2003) reported the existence of dense marker regions on chromosome 18 covering 162.6 cM with 37 loci. A fiber length QTL was located on chromosome 18 (Kohel et al. 2001), as were QTLs for fiber strength and fiber color yellowness (Paterson et al. 2003).

Although MAS with DNA markers linked to important traits has progressed somewhat slowly in cotton, further investigation into the details of the cotton genome, along with the development of more portable DNA markers and a reliable framework linkage map, will accelerate MAS approaches. The SSR markers identified herein that are significantly associated with a natural leaf defoliation QTL should help breeders to transfer this QTL from the donor line (Listopad Beliy) into highly adapted elite cultivars through MAS. This should have a significant economic as well as ecological impact.

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